

Molecular phylogenetic relationships of *Lankanectes corrugatus* from Sri Lanka: endemism of South Asian frogs and the concept of monophyly in phylogenetic studies

Magali DELORME*, Alain DUBOIS*, Joachim KOSUCH** & Miguel VENCES***

* Vertèbres: Reptiles & Amphibiens, USM 0602 Taxonomie & Collections,
Département de Systématique & Evolution Muséum National d'Histoire Naturelle,
25 rue Cuvier, 75005 Paris, France
<delorme@mnhn.fr>, <dubois@mnhn.fr>

** Institut für Zoologie, Abteilung Ökologie, Universität Mainz, Saarstraße 21, 55099 Mainz, Germany

*** Zoological Museum, University of Amsterdam, Mauritskade 61,
1092 AD Amsterdam, The Netherlands

For more than fifteen years, the frog genus *Limnonectes* (Ranidae, Dicroglossinae, Limnodynastini) was considered to contain more than 40 South-East Asian species, and a single species from Sri Lanka, *L. corrugatus*. We analysed 1198 base pairs of the mitochondrial 12S and 16S rRNA genes in *L. corrugatus*, in representatives of most major subgroups of *Limnonectes*, and in several genera thought to be related to this genus. The data allow to significantly exclude a relationship of the Sri Lankan species to South-East Asian *Limnonectes*; instead, it seems clustered with species of *Rana* and *Nyctibatrachus*, which supports the previous recognition of the monotypic genus *Lankanectes* for *L. corrugatus*. The morphological specializations of this species confirm that it may be the only known representative of an additional major ranid lineage (Lankanectinae) endemic to South Asia, an area of high importance as center of basal diversity and endemism of this family. Our data also suggest some comments on the generic taxonomy in the Limnodynastini tribe of the Dicroglossinae. By contradicting previous statements on the monophyly of *Limnonectes*, they also point to a general terminological problem in phylogenetic studies. We propose to use the term *homophyletic* to refer to groups in which the available data do not contradict holophyly but in which taxon sampling is still incomplete or uncertain.

INTRODUCTION

The amphibian fauna of South Asia, that is, India and Sri Lanka, contains an important number of endemic taxa at deep phylogenetic levels. This distinctness of South Asian frogs was already highlighted by BLOMMERS-SCHLÖSSER (1993) who erected the new subfamilies

Indiraninae (now a synonym of Ranixalinae) and Nyctibatrachinae for the endemic Indian genera *Nyctibatrachus* and *Indirana*. The spectacular discovery of the endemic Nasikabatrachidae further confirmed the biogeographic importance of this region (BIJU & BOSSUYT, 2003). The Indian plate had been drifting northwards since its separation from Madagascar 88 million years ago (BARRON et al., 1981, STOREY, 1995; STOREY et al., 1995), and several lineages of frogs may have dispersed out of India into other regions of Asia (DUELLMAN & TRUEB, 1985; BOSSUYT & MILINKOVITCH, 2001). However, surprisingly few phylogenetic studies have focused on South Asian ranids in the past (e.g., BLOMMERS-SCHLÖSSLER, 1993), and only in recent times have some data become available (BOSSUYT & MILINKOVITCH, 2000, 2001, VENCES et al., 2000c, KOSLICH et al., 2001). ROFLANTS et al. (2004) emphasized the deep evolutionary history of several South Asian lineages in the family Ranidae, many of which might be crucial to determine relationships in this family and, in a wider context, in the superfamily Ranoidea. Among the endemic South Asian genera or subgenera which may yield new insights into ranoid biogeography are the Indian microhylid *Melanobatrachus*, the Indian ranids *Climotarsus*, *Indurana*, *Micrixalus*, *Mimivarya*, *Nyctibatrachus* and *Sphaerotheca*, and the Sri Lankan ranid *Nannophrys* (DUBOIS, 1992, 2003, DUBOIS et al., 2001).

Considering the high degree of homoplastic morphological adaptations in frogs, molecular methods have proved to be a useful tool to uncover phylogenetic relationships undisturbed from possible convergent similarities (e.g., HAY et al., 1995; VENCES et al., 2000a). Of the South Asian endemics, so far no published data are available on *Climotarsus*, *Melanobatrachus* and *Mimivarya*, the position of *Indirana*, *Micrixalus* and *Nyctibatrachus* is basically unsolved (BOSSUYT & MILINKOVITCH, 2000, 2001, VENCES et al., 2000c, ROFLANTS et al., 2004); and *Nannophrys* and *Sphaerotheca* proved to be related to the widely distributed genera *Euphylyctis*, *Fejervarya* and *Hoplobatrachus* (BOSSUYT & MILINKOVITCH, 2000, VENCES et al., 2000a,c; KOSLICH et al., 2001). However, as ranoid taxonomy is still largely unsolved, the generic attribution of South Asian species is not in all cases certain.

Another Sri Lankan species of unclarified phylogenetic relationships was described by PEILERS (1863) as *Rana corrugata*. This species was included by BOULENGER (1920) in his section *Ranae kuhlianae* of the genus *Rana*, so that DUBOIS (1981), when he erected *Limnonectes* as a subgenus of *Rana*, and later (1987, 1992) as a distinct genus, included it in this group. Since then, the species has been named *Limnonectes corrugatus* in several works (e.g., DUTTA & MANAMENDRA ARACHCHI, 1996; DUTTA, 1997). However, DUBOIS & OHLER (2001) pointed to morphological characters that exclude this species from *Limnonectes*, and erected for it the monotypic genus *Lankanectes*.

The genus *Limnonectes* as currently understood (e.g., OHLER & DUBOIS, 1999, DUBOIS & OHLER, 2000, 2001, EMERSON et al., 2000; DUBOIS, 2003; EVANS et al., 2003) contains a number of South-East Asian species. Some of these have fangs in the front of their mandibles, so that these species have been named "fanged frogs". They served as a model group to understand the evolution of several traits such as reduction of vocal sacs (EMERSON & VORIS, 1992, EMERSON & BIRRIAN, 1993, EMERSON & WARD, 1998) and to test biogeographical hypotheses at the interface of the Oriental and Australian zones (EVANS et al., 2003). *Limnonectes* has been claimed to constitute a monophyletic group (EMERSON et al., 2000, EVANS et al., 2003), but molecular studies failed to place *L. corrugatus* in a clade with the South-East Asian *L. kuhlii*, type-species of *Limnonectes* (BOSSUYT & MILINKOVITCH, 2000, VENCES et al., 2000c).

Table 1 Species of *Limnometes* and putatively related genera included in this study, their distribution and their allocation to groups or clades proposed by previous authors (1) Taxonomic allocation of "fanged frogs" according to DUBOIS (1992), OHLER & DUBOIS (1999) and DUBOIS & OHLER (2000) E, subgenus *Limnometes* (*Elachyglossa*), Lg, *grunniens* group of the subgenus *Limnometes* (*Limnometes*), Lk, *kuhlii* group of the subgenus *Limnometes* (*Limnometes*), Lm, *microdiscus* group of the subgenus *Limnometes* (*Limnometes*), T, genus *Taylorana* (2) Allocation of "fanged frogs" to subclades 1a, 1b, 2, 3 or 4 of the genus *Limnometes* according to EMERSON et al. (2000) and EVANS et al. (2003).

Species	Taxonomic allocation (1)	Cladistic allocation (2)	Distribution
<i>Fejervarya cancrivora</i>	-	-	China, Indochina, Indonesia, Malaysia
<i>Fejervarya limnocharis</i>	-	-	Indochina, Indonesia, Malaysia
<i>Hoplobatrachus chinensis</i>	-	-	China, Indochina, Indonesia, Malaysia
<i>Limnometes blythii</i>	Lg	4	Indochina, Indonesia, Malaysia
<i>Limnometes goldenstolpei</i>	E	1a	Indochina
<i>Limnometes kuhlii</i>	Lk	1b	Indochina, Indonesia, Malaysia
<i>Limnometes macrocephalus</i>	Lg	3	Philippines
<i>Limnometes paramacrodon</i>	Lg	4	Indonesia, Malaysia
<i>Limnometes woodworthii</i>	Lm	3	Philippines
<i>Taylorana hascheana</i>	T	1a	Indochina, Indonesia
<i>Lankanectes corrugatus</i>	-	-	Sri Lanka

Limnometes is rather species-rich with currently about 50 recognized species but probably many more indeed (EVANS et al., 2003), and several subclades have been identified in this clade (EMERSON et al., 2000, EVANS et al., 2003). However, as these studies did not include *L. corrugatus*, the relationships between this Sri Lankan species and the South-East Asian *Limnometes* remained unclarified. Recently, ROELANTS et al. (2004) included *Lankanectes corrugatus* and two species of *Limnometes* in a molecular phylogenetic analysis, which supported the exclusion of the former species from *Limnometes*.

The aim of this paper is to test more comprehensively if the Sri Lankan species is phylogenetically related to *Limnometes* of South-East Asia or if it may be a representative of an endemic South Asian lineage, using a larger taxonomic sampling than in ROELANTS et al. (2004). For this purpose we analyzed mitochondrial DNA sequences of this species and of representatives of several groups (tab. 1) of *Limnometes* sensu DUBOIS & OHLER (2000) and of three genera, which previously had been included in that genus (*Fejervarya*, *Hoplobatrachus* and *Taylorana*).

MATERIALS AND METHODS

Tissue samples (muscle or liver, either fresh or preserved in 98% ethanol) were available from 25 ranoid species. DNA was extracted using QIAmp tissue extraction kits (Qiagen). We amplified two fragments of 12S rRNA gene (417 pb and 470 pb). The original couple of primers are based on the sequence of 12S of *Rana catesbeiana* (Genbank accession number M18125) L7 (5' -TTT GGT CCT AGC CTT ATT ATC -3') with H424 (5' -GGC ATA GTG GGG TAT CTA ATC -3'), and L428 (5' -CTT AAA ACC CAA AGG ACT TGA -3')

Table 2 – Specimens examined in the present study. Collection abbreviations used: FD, Forest Departement, Bangkok; FMNH, Field Museum, Chicago; KUHE, Graduate School of Human and Environmental Studies, Kyoto University Japon; MNHN, Muséum National d'Histoire Naturelle, Paris; MV, field number of Michael Veith, specimens to be catalogued in the Field Museum, Chicago; SI, Smithsonian Institution, WHT, Wildlife Heritage Trust, Colombo; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn; ZMB, Zoologisches Museum der Universität, Berlin; ZSM, Zoologische Staatssammlung, München. Genbank accession numbers marked with an asterisk refer to sequences obtained by other authors.

Species	Origin 16S / 12S	Collection number 16S	Genbank accession 16S	Collect on number 12S	Genbank accession 12S
<i>Buergeria buergeri</i>	-	KUHE 26541		KUHE 26541	
<i>Bufo melanostictus</i>	-		AF249061		U52721
<i>Ceratobatrachus guentheri</i>	Solomon Islands	ZMFK 50484		ZMFK 50484	
<i>Chaparana fanjansi</i>	Sapa, Vietnam	MNHN 1999 5818		MNHN 1999 5818	
<i>Eleutherodactylus cuneatus</i>	-		X86310		Y10944
<i>Euphyctes cyanophlyctis</i>	Cochin, India / Sri Lanka	MNHN 2000.650	AY014366	WHT 0043C	
<i>Feyervarya cancrivora</i>	Sumatra	FMNH 256692	AY014380	FMNH 256692	
<i>Feyervarya limaoharis</i>	Laos / Laos	MNHN 1997 3932	AF215416	MNHN 1997 5608	
<i>Hoplobatrachus chinensis</i>	Laos / Laos	MNHN 1997 4900	AY014368	MNHN 1997 5691	
<i>Ingerana baluensis</i>	Malaysia	FMNH 231085		FMNH 231085	
<i>Lankaneetes corrugatus</i>	Sri Lanka	WHT 0020C		WHT 0020C	
<i>Limnonectes blythii</i>	Phang Nga, Thailand	MNHN 1998 19		MNHN 1998.19	
<i>Limnonectes goldenstolpet</i>	Vietnam	MNHN 1998 4150		MNHN 1998.4150	
<i>Limnonectes kuhlii</i>	Laos / Phang Nga, Thailand	MNHN 1997 3904	AF215415	FD P921	
<i>Limnonectes macrocephalus</i>	Leyte, Philippines	MV 365		MV 365	
<i>Limnonectes woodworthi</i>	Leyte, Philippines	MNHN 2000 612		MNHN 2000 612	
<i>Occidopygia lima</i>	Philippines / Laos	ZMB 50910	AF215398	MNHN 1999 6113	
<i>Nyctibatrachus</i> sp.	Ootacamund, India		AF215397		AF215199
<i>Nyctibatrachus cf. alliciae</i>	-		AF249018		AF249063
<i>Nyctibatrachus major</i>	-		AF249017		AF249052
<i>Paa bourreil</i>	Sapa, Vietnam	MNHN 1999 5861		MNHN 1999 5861	
<i>Polydactylus equei</i>	Sri Lanka	WHT 0036C		WHT 0036C	
<i>Rana catesbeiana</i>	-		X12841		MIRC12S
<i>Rana temporaria</i>	Koblenz, Germany / France	ZFMK 69883	AF124135	MNHN 1998 5	
<i>Sphaerotheca phalaena</i>	Myanmar	SI 520491		SI 520491	
<i>Taratorhinus huxleyana</i>	Vietnam	MNHN 1997 5355		MNHN 1997 5355	

with H898 (5' ACC ATG TTA CGA CTT GCC TCT 3'). For the 16S rRNA gene, we amplified one fragment using the primers (of PALUMBI et al. 1991) 16SA-L (light chain; 5' CGC CTG TTT ATC AAA AAC AT 3') and 16SB-H (heavy chain; 5' - CCG GTC TGA ACT CAG ATC ACG T - 3'). We followed the PCR conditions as given in VENCES et al. (2000b) and the PCR products were purified and sequenced using automatic sequencers (ABI 377 or CEQ 2000 Beckmann). The sequences (see tab 2 for Genbank accession numbers) were aligned using the program Se-Al (RAMBALI, 1995), and by taking account of the secondary structure of molecules (KJER, 1995, 1997). Gapped positions were excluded from analysis. Two outgroups and three ingroup sequences (*Eleutherodactylus cuneatus*, *Bufo melanostictus*, *Rana catesbeiana*, *Nyctibatrachus major*, *Nyctibatrachus cf. alliciae*) from Genbank were further added to the alignment.

To assess whether the different gene fragments could be submitted to combined analysis, we tested all possible combinations using the partition homogeneity test (parsimony method of FARRIS et al. 1995), as implemented in PAUP*, version 4b8 (SWOFFORD, 2001). Prior to

phylogenetic reconstruction, we explored which substitution model fits our sequence data the best using the program MODELTEST (POSADA & CRANDALL, 1998). The presence of a significant phylogenetic signal was estimated using the permutation-tailed-probability (PTP) test with 100 replicates implemented in PAUP*.

Phylogenetic analyses were carried out using PAUP*. We calculated maximum parsimony (MP) and maximum likelihood (ML) trees. In the MP analyses we conducted heuristic searches with initial trees obtained by simple stepwise addition, followed by branch swapping using the TBR (tree bisection-reconnection) routine implemented in PAUP*. Ten random addition sequence replicates were carried out. The ML trees were obtained using heuristic searches, using the substitution model proposed by MODELTEST.

Following HEDGES (1992), 2000 bootstrap replicates (FELSENSTEIN, 1985) were run in the MP analysis whereas only 100 (full heuristic) ML bootstrap replicates were possible because of computational constraints.

Furthermore, we used Bayesian inference in the program MrBayes 2.01 (HUELSENBECK & RONQUIST, 2001). We run four simultaneous Metropolis-coupled Monte Carlo Markov chains for 500,000 generations, sampling a tree every ten generations. The initial set of generations needed before convergence on stable likelihood values ("burnin") was set at 50,000 (10 %) based on empirical evaluation.

RESULTS

A chi-square test did not contradict homogeneity of base frequencies across taxa ($df = 78$, $P > 0.9$). The partition homogeneity test did not reject the null hypothesis of congruence of the included gene fragments (1000 replicates; $P > 0.5$), thus not contradicting their suitability for combination in phylogenetic analysis. The PTP test resulted in a significant difference ($P = 0.01$) between the most parsimonious tree and trees generated from random permutations of the data matrix, demonstrating presence of significant phylogenetic signal. After exclusion of gapped states, of 1122 characters included in the analysis, 504 were constant, 179 variable but parsimony uninformative, and 439 variable and parsimony-informative. Maximum parsimony analysis found one most parsimonious tree (2422 steps, consistency index 0.414, retention index 0.412). MODELTEST proposed a Tamura-Nei substitution model with a gamma shape parameter of 0.433, a proportion of invariable sites of 0.190, and user-defined substitution rates (A-G, 3.7290; C-T, 7.5587; all other rates, 1) and base frequencies (A, 0.3857; C, 0.2267; G, 0.1407; T, 0.2469).

The ML analysis using the settings proposed by MODELTEST resulted in the tree shown in fig. 1. All species of *Limnectes* (excluding *L. corrugatus*) were grouped as a homophyletic group, in which *Taylorana hascheana* was also included. Species of *Fejervaria* (once a subgenus of *Limnectes*) did not directly cluster with *Limnectes*. The included taxa placed by DUBOIS (1992) in the Dicroglossinae (a subfamily of the Ranidae) were a homophyletic lineage, which also included the genera *Paa* and *Chapanana* placed by the latter author in the Panni, a tribe then referred to the Raninae but later transferred into the Dicroglossinae (DUBOIS et al., 2001; DUBOIS, 2003; JIANG & ZHOU, in press). *Lankanectes*

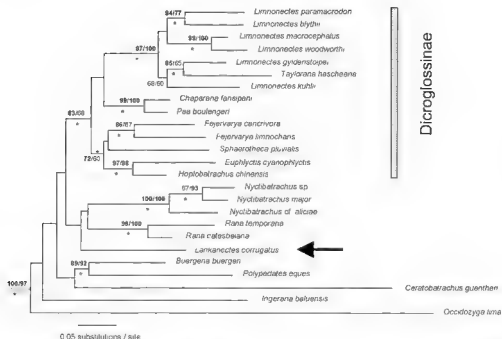


Fig. 1 Maximum likelihood phylogram calculated by PAUP* using a TRN + I + G substitution model selected by MODELTEST, based on 1198 nucleotides of the mitochondrial 12S and 16S rRNA genes. Numbers are bootstrap values (in percent; 100 and 2000 replicates) of maximum likelihood and maximum parsimony analyses. Asterisks mark nodes that received posterior probability values of 99-100% in a Bayesian analysis. Values below 50% are not shown. The arrow marks the Sri Lankan species *Lankaneetes corrugatus* which previously was considered as member of the genus *Limnonectes* in the subfamily Dicroglossinae. *Bufo melanostictus* and *Eleutherodactylus cuneatus* were used as outgroups (not shown).

corrugatus was placed as sister group to a clade containing *Nyctibatrachus* and *Rana*, the type-genus of the Raninae. *Oedipodyga lima* was the outgroup to all other ranoids included, confirming its strong differentiation in the mitochondrial rRNA genes already emphasized by MARMAYOU et al. (2000). Most of these groupings were also found in MP and NJ analyses (not shown) and received moderate to strong bootstrap support (fig. 1).

DISCUSSION

RELATIONSHIPS OF *LANKANEETES CORRUGATUS* AND ENDEMISM IN SOUTH ASIAN ANURANS

Our results confirm again the existence of a well-defined clade Dicroglossinae among the Ranidae, and provide support for at least three subclades in this subfamily, which can

taxonomically be considered as tribes (DUBOIS, 2003). The genera included in the present study were distributed as follows among these lineages (1) *Limnionectini* (*Limnionectes* and *Taylorana*), (2) *Dicroglossini* (*Euphlyctis*, *Fejervarya*, *Hoplobatrachus* and *Sphaerotheca*); (3) *Paini* (*Paa* and *Chaparana*).

Our data provide strong evidence that *Lankanectes corrugatus* does not belong to the *Limnionectini*, let alone to the *Dicroglossinae*. In our analysis this species was instead placed close to *Nyctibatrachus* and *Rana*. However, bootstrap support for this grouping was low. Weak indications for relationships of *Lankanectes* to *Nyctibatrachus* and *Rana* were also apparent from the results of BOSSUYT & MILINKOVITCH (2000) and VENCES et al. (2000c). However, morphologically *Lankanectes* is well distinguished from these genera by several divergent characters such as its forked omosternum (unforked in *Rana*) or the rare pedomorphic presence of a functional lateral-line system in adults (DUBOIS & OHLER, 2001), a character shared with the dicroglossine *Euphlyctis* and the basal genus *Occidozyga* but absent in *Rana* or *Nyctibatrachus*.

The data set of BOSSUYT & MILINKOVITCH (2000) contained almost 2700 nucleotides of mitochondrial and nuclear genes, but their analyses did nevertheless not provide high support for relationships of *Lankanectes* to *Rana* or *Nyctibatrachus*. Furthermore, no indications for close relationships of the species to other South Asian endemics (*Indirana*, *Mictalalus*, *Nannophrys*) have been found (BOSSUYT & MILINKOVITCH, 2000; VENCES et al. 2000c). ROELANTS et al. (2004)'s results, based on a much smaller sample of *Limnionectes* than ours, also show that *L. corrugatus* does not belong in the *Dicroglossinae* clade and does not have any close relation with the *Raninae*. *Lankanectes corrugatus* would be placed in basal position of the *Ranidae* with the genus *Nyctibatrachus*, but no strong support exists for this relation. Therefore we are inclined to assume that *L. corrugatus* is the sole known representative of a further endemic South Asian ranid lineage. This implies recognition of a new genus for this species, which may be at least provisionally placed in a subfamily *Lankanectinae*, of unclear affinities (DUBOIS & OHLER, 2001; DUBOIS, 2003; ROELANTS et al., 2004). These data strongly confirm the importance of South Asia as a center of endemism of basal ranid lineages (BOSSUYT & MILINKOVITCH, 2001; ROELANTS et al., 2004). They also show that much more remains to be learned on the relationships among basal ranid lineages. Certainly, a much larger amount of molecular data is needed before a comprehensive scenario of the evolution of this group can be drawn.

GENERIC TAXONOMY OF LIMNIONECTINI

Incidentally, our results provide additional support to previous data regarding relationships within the South-East Asian *Limnionectini* clade. All South-East Asian species of *Limnionectes* we surveyed were included in a single subclade of the dicroglossine lineage. In this group, *Limnionectes goldenstolpeti* (see OHLER & DUBOIS, 1999) was placed as sister group of *Taylorana hascheana*. The topology of our tree, as well as those of other recent studies (EMERSON et al., 2000; EVANS et al., 2003), indicate paraphyly of the genus *Limnionectes* as currently understood (DUBOIS & OHLER, 2001). This does not necessarily imply that *Taylorana* should be synonymized with *Limnionectes*. The genus *Taylorana* is well-defined by presence of male mating call (absent in *Limnionectes*) and of direct development (TAYLOR,

1962; ÖHLER et al., 1999). This latter character is particularly relevant in anuran generic taxonomy (DUBOIS, 1987, 1988, 2004). According to the precise suggestions of DUBOIS (2004), if confirmed the cladograms referred to would rather suggest that, beside *Taylorana*, three genera at least should be recognized in the *Limnocoetini*: (1) a first one, for which the nomen *Elachyglossa* Andersson, 1916 is available, including the species listed by ÖHLER & DUBOIS (1999) and possibly others such as *Rana lateps* Boulenger, 1882; (2) a second one, that should retain the nomen *Limnocoetes* Fitzinger, 1843, for *L. kuhli* and a few other species; (3) a third one, including most species of the *grunniens* and *microdiscus* groups of DUBOIS (1987: 63) or of the subclades 2, 3 and 4 of EMERSON et al. (2000) and EVANS et al. (2003). No generic nomen has been associated with the latter group until now, but such a nomen might be available. Recent re-interpretation of morphological characters of the species originally described as *Rana delacourii* by ANGEL (1928) and later placed in the subgenus *Chaparana* (*Annandia*) by DUBOIS (1992), now suggests that this species may be closer to *Limnocoetes blythii* than to members of the tribe *Paini* (DUBOIS & ÖHLER, in preparation). As this species is the type-species of *Annandia* Dubois, 1992, the latter nomen might be available for the third genus outlined above. At any rate, until the cladistic relationships of *Rana delacourii* are clarified, it would appear better not to create a generic nomen for the latter group.

"FANGED" FROGS AND THE CONCEPTS OF MONOPHYLY, HOMOPHYLY AND HOLOPHYLY

EMERSON et al. (2000: 136) wrote that "the fanged frogs constitute a monophyletic group" and that "it seems appropriate, in the future, to refer to these frogs as members of the genus *Limnocoetes*". While doing so, however, they did not provide a list of taxa that they referred to this genus, so that one can infer that they probably adopted DUBOIS's (1992) concept of the latter, thus including the fang-bearing species *Lankacoetes corrugatus* (as *Limnocoetes corrugatus*)

However, our data once again show that the latter species is not a member of *Limnocoetes*, and that this genus as it has been understood until the work of DUBOIS & ÖHLER (2001) is not monophyletic. Despite this apparent contradiction, the statement of EMERSON et al. (2000) regarding monophyly of "fanged" frogs was not incorrect: actually, all species studied by these authors appeared as a clade in their molecular analysis, and were not para- or polyphyletic relative to the other taxa studied. This problem is a more general one in phylogenetic studies, especially those relying on molecular data. In many cases, because of material limitations, such studies can include only some of the species of the group whose monophyly is to be tested. However, as noted by BOSSUYT & DUBOIS (2001: 4), the large impact of species sampling on cladistic analysis should not be underestimated. This has long been known for cladistic studies based on morphology. "Ideally, all known taxa of a group should be included in analysis, since omission can lead to misinterpretation of transformation series (...) and of relationships (...)" (ARNOLD, 1981: 29).

Part of the confusion is mostly semantic, being rooted in the use of the unclear term *monophyletic*. This term was introduced in scientific literature by HAECKEL (1868) as an antonym to *polyphyletic*, but HENNIG (1950) redefined it as an antonym to both *polyphyletic* and *paraphyletic*, a new concept introduced by him. The Hennigian definition of a monophyletic group, adopted by many current authors, can be worded as follows: "A group that

includes a common ancestor and all of its descendants" (WILEY, 1981: 84). In logical terms, this means that a monophyletic group has two qualifications, uniqueness (non-polyphyly) and completeness. Like all double concepts, this can be sorted in two distinct concepts, for which, in order to avoid the confusions linked to the use of the unclear term *monophyletic*, two distinct terms have been proposed: *homophyletic* (DUBOIS, 1986, 1988) for unique or non-polyphyletic, and *holophyletic* (ASHLOCK, 1971) for unique and complete. Many authors now use the term *monophyletic* for the latter concept, but then, if they claim that a group is unique and complete, they should provide the complete list of included taxa, at least among the taxa then known and recognized as valid by zoologists in the taxon (family or even higher taxon) under study.

Because many clades certainly contain extinct species, sometimes in considerable number, whose fossils will never be found, absolute completeness of sampling of taxa will remain impossible in many zoological groups. Even the goal of completeness of sampling of extant taxa is often unrealistic because, despite the ongoing and even accelerating high rate of discovery of new species, it is clear that many or most extant animal species are not even known (and certainly not taxonomically described) yet. But a different thing is to realise that, among the species that we have discovered and described, stating that a group is complete means that we have identified all those that are members of a given clade. This will be done only when all species have been properly studied with the techniques (molecular, morphological or other) that we use to allocate them to clades. The example of *Lankanectes* shows that any single species, once seriously studied, may contradict our previous hypotheses. In this case, one can argue that its strange geographical distribution might have indicated long ago that *L. corrugatus* was an intruder in *Limnnectes*, but this is not always the case. Thus, in the same frog group, the case of the species *Rana delacouri* mentioned above, if confirmed, would illustrate a rather frequent situation in which neither geographical distribution nor overall morphology had allowed to suspect wrong cladistic allocation of a species: in such cases, the proper study of a single species may have nomenclatural implications, e.g. if this species is the type of a nominal genus.

Therefore, in many cladistic analyses, especially molecular, as only a partial list of taxa has been actually studied, it would be more prudent and exact to state that the group composed of these studied taxa is *homophyletic*, i.e. non-polyphyletic, without going further in inferring the actual cladistic position of taxa whose existence is known but that were not examined in the study. Only when all known potentially related taxa have been properly studied and allocated a place in the cladogram is it justified to state that a group appears non-polyphyletic and complete, i.e. "monophyletic" or, better as fully unambiguous, *holophyletic*.

RÉSUMÉ

Depuis plus de 15 ans, le genre *Limnnectes* (Ranidae, Dicroglossinae, Limnnectini) a regroupé plusieurs dizaines d'espèces du Sud Est de l'Asie, ainsi qu'une espèce isolée provenant du Sri Lanka, *L. corrugatus*. Nous avons analysé 1198 paires de base des gènes ARNr mitochondriaux 12S et 16S de *L. corrugatus*, des représentants de tous les principaux sous-groupes de *Limnnectes* et de plusieurs genres qui semblent proches. Les données ont

permis d'exclure clairement l'espèce du Sri Lanka des *Limnometes* du Sud-Est de l'Asie. De plus, celle-ci semble se rapprocher des genres *Rana* et *Nyctibatrachus*, ce qui étaye la reconnaissance récente du genre monotypique *Lankanectes* pour *L. corrugatus*. Les spécialisations morphologiques de cette espèce confirmant qu'elle serait la seule représentante connue d'une lignée de Ranides endémique de l'Asie du Sud, une région de grande importance comme centre de diversité et d'endémisme de cette famille. Nos données suggèrent également quelques commentaires sur la taxinomie générique de la tribu des Limnometini. En contradiction avec les précédents résultats sur le monophylétisme de *Limnometes*, elles mettent l'accent sur un problème général de terminologie dans les études phylogénétiques. Nous proposons d'utiliser le terme *homophylétique* pour des groupes pour lesquels les données disponibles ne sont pas contradictoires avec l'hypothèse de monophylétisme, mais dont le contenu est encore incomplet ou incertain.

ACKNOWLEDGMENTS

For the help received in Sri Lanka, we thank Ronan Pethiyagoda and Kelum Manamendra-Arachchi. We are grateful to Annemarie Ohler, Stéphane Grosjean and Renaud Boistel (Paris) and to Alan Resetar (Chicago) for providing tissues of several Asian and Oriental frog taxa. Franco Andreone provided useful comments on the manuscript. This work was supported by the PPF "Faune et flore du sud-est asiatique" of the Paris Museum.

LITERATURE CITED

- ANGELL, M. F., 1928. Sur une espèce nouvelle de grenouille du Tonkin, appartenant au genre *Rana*. *Bull. Mus. nat. Hist. nat.*, **34**: 319-320.
- ARNOLD, E. N., 1981. Estimating phylogenies at low taxonomic levels. *Zool. Syst. Evol.-forsch.*, **19**: 1-35.
- ASHLOCK, P. D., 1971. - Monophyly and associated terms. *Syst. Zool.*, **20**: 63-69.
- BARRON, E. J., HARRISON, C. G. A., SLOAN, J. L., II & HAY, W. W., 1981. Paleogeography, 180 million years ago to the present. *Ecol. Geol. Helveticae*, **74**: 443-470.
- BIJU S. D. & BOSSUYT, F., 2003. New frog family from India reveals an ancient biogeographical link with the Seychelles. *Nature*, **425**: 711-714.
- BLOMMERS-SCHLÖSSER, R. M. A., 1993. Systematic relationships of the Mantellinae Laurent 1946 (Anura Ranoidea). *Ethol. Ecol. Evol.*, **5**: 199-218.
- BOSSUYT, F. & DU BOIS, A., 2001. A review of the frog genus *Philautus* Gistel, 1848 (Amphibia, Anura, Ranidae, Rhacophoridae). *Zeylanica*, **6** (1): 1-112.
- BOSSUYT, F. & MIHAIKOVITICH, M. C., 2000. Convergent adaptive radiations in Madagascan and Asian rain forest frogs reveal covariation between larval and adult traits. *Proc. Natl. Acad. Sci. USA*, **97** (12): 6585-6590.
- , 2001. Amphibians as indicators of early Tertiary "Out-of-India" dispersal of vertebrates. *Science*, **292**: 93-95.
- BULLINGER, G. A., 1920. A monograph of the South Asian, Papuan, Melanesian, and Australian frogs of the genus *Rana*. *Rec. Indian Mus.*, **20**: 1-226.
- DUBOIS, A., 1981. - Liste des genres et sous-genres nominaux de Ranoidea (Amphibiens, Anoures) du monde, avec identification de leurs espèces-types, conséquences nomenclaturales. *Mont. zool. ital.*, (n.s.), **15** (suppl.): 225-284.

- 1986. – A propos de l'emploi controversé du terme "monophylétique": nouvelles propositions. *Bull. mens. Soc. linn. Lyon*, **55**: 248-254.
- 1987. – Miscellanea taxinomica batrachologica (I). *Alytes*, **5** (1-2): 7-95.
- 1988. – The genus in zoology: a contribution to the theory of evolutionary systematics. *Mém. Mus. natn. Hist. nat.*, (A), **140**: 1-123.
- 1992. – Notes sur la classification des Ranidae (Amphibiens Anoures). *Bull. mens. Soc. linn. Lyon*, **61**: 305-352.
- 1999. – Miscellanea nomenclatorica batrachologica. 19. Notes on the nomenclature of Ranidae and related groups. *Alytes*, **17** (1-2): 81-100.
- 2003. – True frogs (Ranidae). In: M. HUTCHINS, W. E. DUELLMAN & N. SCHLAGER (ed.), *Grzimek's animal life encyclopedia*, 2nd edition, Volume **6**, *Amphibians*, Farmington Hill, Michigan, Gale Group: 245-264.
- 2004. – Developmental pathway, speciation and supraspecific taxonomy in amphibians. 2. Developmental pathway, hybridizability and generic taxonomy. *Alytes*, **22** (1-2): 38-52.
- DUBOIS, A. & OHLER, A., 2000. – Systematics of *Fejervarya limnocharis* (Gravenhorst, 1829) (Amphibia, Anura, Ranidae) and related species. 1. Nomenclatural status and type-specimens of the nominal species *Rana limnocharis* Gravenhorst, 1829. *Alytes*, **18** (1-2): 15-50.
- 2001. – A new genus of Ranidae (Amphibia, Anura) from Sri Lanka. *Alytes*, **19** (2-4): 81-106.
- DUBOIS, A., OHLER, A. & BIJU, S. D., 2001. – A new genus and species of Ranidae (Amphibia, Anura) from southern India. *Alytes*, **19** (2-4): 53-79.
- DUELLMAN, W. E. & TRUEB, L., 1985. – *Biology of amphibians*. New York, McGraw-Hill, "1986": i-xix + 1-670.
- DUTTA, S. K., 1997. – *Amphibians of India and Sri Lanka. (checklist and bibliography)*. Bhubaneswar, Odyssey Publishing house: [i-iii] + i-xiii + 1-342 + i-xxii.
- DUTTA, S. K. & MANAMENDRA ARACHCHI, K., 1996. – *The amphibians fauna of Sri Lanka*. Colombo, Wildlife Heritage Trust of Sri Lanka: 1-232.
- EMERSON, S. B. & BERRIGAN, D., 1993. – Systematics of Southeast Asian ranids: multiple origins of voicelessness in the subgenus *Limnonectes* (Fizinger). *Herpetologica*, **49** (1): 22-31.
- EMERSON, S. B., INGER, R. F. & ISKANDAR, D., 2000. – Molecular systematics and biogeography of the fanged frogs of Southeast Asia. *Mol. Phyl. Evol.*, **16**: 131-142.
- EMERSON, S. & VORIS, H., 1992. – Competing explanations for sexual dimorphism in a voiceless Bornean frog. *Funct. Ecol.*, **6**: 654-660.
- EMERSON, S. & WARD, R., 1998. – Male secondary sexual characters, sexual selection, and molecular divergence in fanged ranid frogs of Southeast Asia. *Zool. J. Linn. Soc.*, **122**: 537-553.
- EVANS, B. J., BROWN, R. F., MCGUIRE, J. A., SUPRIATNA, J., ANDAYANI, N., DIEMOS, A., ISKANDAR, D., MELNICK, D. J. & CANNATELLA, D. C., 2003. – Phylogenetics of fanged frogs: testing biogeographical hypotheses at the interface of the Asian and Australian faunal zones. *Syst. Biol.*, **52** (6): 794-819.
- FARRIS, J. S., KÄLLERSJÖ, M., KLUGE, A. G. & BULT, C., 1995. – Testing significance of incongruence. *Cladistics*, **10**: 315-319.
- FELSENSTEIN, J., 1985. – Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**: 783-791.
- HAECKEL, E., 1868. – *Natürliche Schöpfungsgeschichte*. Berlin, Reimer: i-xvi + 1-568, front + pl. 1-8 + p. 204 b-c.
- HAY, J. M., RUVINSKY, I., HEDGES, S. B. & MAXSON, L. R., 1995. – Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. *Mol. Biol. Evol.*, **12** (5): 928-937.
- HEDGES, S. B., 1992. – The number of replications needed for accurate estimation of the bootstrap P-value in phylogenetic studies. *Mol. Biol. Evol.*, **9**: 366-369.
- HENNIG, W., 1950. – *Grundzüge einer Theorie der phylogenetischen Systematik*. Berlin, Deutscher Zentralverlag: 1-370.
- HUELSENBECK, J. P. & RONQUIST, F., 2001. – MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**: 754-755.
- JIANG, J.-P. & ZHOU, K. Y., in press. – Phylogenetic relationships among Chinese ranids inferred from sequence data set of 12s and 16s rDNA. *Herp. J.*

- KJER, K. M., 1995. – Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Mol. Phyl. Evol.*, **4** (3): 314-330.
- 1997. – An alignment template for amphibian 12S rRNA, domain III: conserved primary and secondary structural motifs. *J. Herp.*, **31**: 599-604.
- KOSUCH, J., VENCES, M., DUBOIS, A., OHLER, A. & BÖHME, W., 2001. – Out of Asia: mitochondrial DNA evidence for an Oriental origin of tiger frogs, genus *Hoplobatrachus*. *Mol. Phyl. Evol.*, **21**: 398-407.
- MARMAYOU, J., DUBOIS, A., OHLER, A., PASQUET, E. & TILLIER, A., 2000. – Phylogenetic relationships in the Ranidae. Independent origin of direct development in the genera *Philautus* and *Taylorana*. *C. r. Acad. Sci. Paris, Sciences de la Vie*, **323**: 287-297.
- OHLER, A. & DUBOIS, A., 1999. – The identity of *Elachyglossa gyldenstolpei* Andersson, 1916 (Amphibia, Ranidae), with comments on some aspects of statistical support to taxonomy. *Zoologica scripta*, **28** (3-4): 269-279.
- OHLER, A., GROSJEAN, S. & HOYOS, J. M., 1999. – Observation de la construction d'un nid chez *Taylorana hascheana* (Anura: Ranidae). *Revue fr. Aquariol.*, **26**: 67-70.
- PALUMBI, S. R., MARTIN, A., ROMANO, S., McMILLAN, W. O., STICE, L. & GRABOWSKI, G., 1991. – *The simple fool's guide to PCR, Version 2.0*. Privately published document compiled by S. Palumbi, Dept. Zoology, Univ. Hawaii, Honolulu, HI, 96822.
- PETERS, W., 1863. – Mittheilung über eine neue Schlangengattung, *Styporhynchus*, und verschiedene andere Amphibien des zoologischen Museums. *Monatsb. königl. Akad. Wiss. Berlin*, **1863**: 399-413.
- POSADA, D. & CRANDALL, K. A., 1998. – MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**: 817-818.
- RAMBAUT, A., 1995. – *Se-Al Sequence Alignment Editor Version 1. dl*. Department of Zoology, University of Oxford.
- ROELANTS, K., JIANG, J. & BOSSUYT, F., 2004. – Endemic ranid (Amphibia: Anura) genera in southern mountain ranges of the Indian subcontinent represent ancient frog lineages: evidence from molecular data. *Mol. Phyl. Evol.*, **31**: 730-740.
- STOREY, B. C., 1995. – The role of mantle plumes in continental breakup: case histories from Gondwanaland. *Nature*, **377**: 301-308.
- STOREY, M., MAHONEY, J. J., SAUNDERS, A. D., DUNCAN, R. A., KELLEY, S. P. & COFFIN, M. F., 1995. – Timing of hot spot-related volcanism and the breakup of Madagascar and India. *Science*, **167**: 852-855.
- SWOFFORD, D. L., 2001. – *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland, Sinauer Associates.
- TAYLOR, E. H., 1962. – The amphibian fauna of Thailand. *Univ. Kansas Sci. Bull.*, **43**: 265-599.
- VENCES, M., GLAW, F., KOSUCH, J., DAS, I. & VEITH, M., 2000a. – Polyphyly of *Tomopterna* (Amphibia: Ranidae) based on sequences of the mitochondrial 16S and 12S rRNA genes, and ecological biogeography of Malagasy relict amphibian groups. In: W. R. LOURENÇO & S. M. GOODMAN (ed.), *Diversité et endémisme de Madagascar*, Paris, Mémoires de la Société de Biogéographie: 229-242.
- VENCES, M., KOSUCH, J., LÖTTERS, S., WIDMER, A., JUNGFER, K.-H., KÖHLER, J. & VEITH, M., 2000b. – Phylogeny and classification of poison frogs (Amphibia: Dendrobatidae), based on mitochondrial 16S and 12S ribosomal RNA gene sequences. *Mol. Phyl. Evol.*, **14**: 34-40.
- VENCES, M., WANKE, S., ODIERNA, G., KOSUCH, J. & VEITH, M., 2000c. – Molecular and karyological data on the south Asian ranid genera *Indrana*, *Nyctibatrachus* and *Nannophrys* (Anura: Ranidae). *Hamadryad*, **25**: 75-82.
- WILLY, E. O., 1981. – *Phylogenetics. The theory and practice of phylogenetic systematics*. New York, Wiley: i-xv + 1-439.

Corresponding editor: FRANCO ANDREONE.

